

IN THE SPECIFICATION:

Please delete the first paragraph on page 1 with the following:

"This application is a continuation of U.S. Serial No. 08/924,287, filed September 5, 1997, now U.S. Patent No. 6,699,838, now allowed, which is a continuation-in-part of U.S. Serial No. 08/851,350 filed May 5, 1997, now U.S. Patent No. 6,057,122 which is a continuation-in-part of U.S. Serial No. 08/832,087 filed April 3, 1997, now U.S. Patent No. 5,981,484, which is a continuation-in-part of U.S. Serial No. 08/643,219 filed May 3, 1996, now U.S. Patent No. 5,801,146."

Please amend page 4, lines 33-34 as follows:

"FIG. 5 shows a map of expression vector pHil-D8 containing a leader sequence for recombinant protein secretion (SEQ ID NOS: 50 and 51, respectively)."

Please amend page 40, lines 27 – page 42, line 11 as follows:

"N-Ac-Val-Leu-Leu-Pro-Asp-Val-Glu-Thr-Pro-Ser-Glu-Glu-Asp-NH₂ (SEQ ID NO: 41)

An amide peptide synthesis column (Applied Biosystems) was placed in the peptide synthesis column position of a Perkin Elmer/Applied Biosynthesis "Synergy" peptide synthesizer, and the following synthetic sequence was used:

1. Solvating the resin with DMF for about 5 minutes;
2. Deblocking the Fmoc group from the α -N-terminal of the resin-bound amino acid using 20% piperidine in DMF for about 15 minutes;
3. Washing the resin with DMF for about 5 minutes;
4. Activating the α -C-terminal of amino acid No. 1 (Fmoc-Asp(β -O^tBu), 25 μ mol) using a 0.2 M solution of HBTU (25 μ mol) and HOBT (25 μ mol) in DMSO-NMP (N-methylpyrrolidone) and a 0.4 M solution of diisopropylethylamine (25 μ mol) in DMSO-NMP and coupling the activated amino acid to the resin;
5. Coupling the activated Fmoc-protected amino acid (prepared in step 5) to the resin-bound amino acid (prepared in step 2) in DMF for about 30 minutes;
6. Washing with DMF for 5 minutes;
7. Repeating steps 3 through 6 with the following amino acids:

No. Amino Acid

2. Fmoc-Glu(γ -O^tBu)
3. Fmoc-Glu(γ -O^tBu)
4. Fmoc-Ser(^tBu)
5. Fmoc-Pro

6. Fmoc-Thr(^tBu)
7. Fmoc-Glu(γ -O^tBu)
8. Fmoc-Val
9. Fmoc-Asp(β -O^tBu)
10. Fmoc-Pro
11. Fmoc-Leu
12. Fmoc-Leu
13. Fmoc-Val

8. Coupling acetic acid to the α -N-terminal of the resin-bound peptide *via* the conditions of steps 4 and 5.

9. Washing the resin with THF for about 5 minutes to remove DMF and shrink the resin, then drying the resin with argon for 10 minutes and nitrogen for 10 minutes more to provide clean, resin-bound peptide.

10. Cleaving of the peptide from the resin with concomitant deprotection of amino acid side chains by stirring with cleavage reagent (freshly-prepared thioanisole (100 μ L), water (50 μ L), ethanedithiol (50 μ L) and trifluoroacetic acid (1.8 mL) mixed in the above order at -5 °C to -10 °C) at 0 °C for 10-15 minutes and then at ambient temperature for an additional 1.75 hours (plus an additional 0.5 hour for each Arg(Pmc), if present). The amount of cleavage reagent used was determined by the following formula:

<u>weight of resin with bound peptide (mg)</u>	<u>amount of cleavage reagent (μL)</u>
0-10	100
10-25	200
25-50	400
50-100	700
100-200	1200

11. Filtering and rinsing the ~~product~~ product with neat trifluoroacetic acid, adding the filtrate in 0.5 mL portions to a centrifuge tube containing about 8 mL of cold diethyl ether, centrifuging and decanting and repeating the process until all of the peptide precipitated (if the peptide did not precipitate upon addition to ether, the mixture was extracted with aqueous 30% aqueous acetic acid (3x1 mL), and the combined aqueous extracts were lyophilized to provide the product).

12. Using the peptide crude or purifying the peptide by HPLC using a 7 μ m Symmetry Prep C18 column (7.8 x 300 mm) with solvent mixtures varying in a gradient from 5% to 100% acetonitrile- (water, 0.1% TFA) over a period of 50 minutes followed by lyophilizing to provide 35 mg of N-Ac-Val-Leu-Leu-Pro-Val-Glu-Thr-Pro-Ser-Glu-Glu-Asp-NH₂ (SEQ ID NO: 41).”

Please amend page 42, line 14 – page 43, line 3 to read as follows:

“N-Ac-Met-Phe-Gly-Asn-Gly-Lys-Gly-Tyr-Arg-Gly-Lys-Arg-Ala-Thr-Thr-Val-Thr-Gly-Thr-Pro-NH₂ (SEQ ID NO: 42)

The title compound was prepared using the synthetic sequence described in Example 1 and using Fmoc-Pro as amino acid No. 1. The following amino acids were added using the conditions indicated:

<u>No.</u>	<u>Amino Acid</u>
2.	Fmoc-Thr(tBu)
3.	Fmoc-Gly
4.	Fmoc-Thr(tBu)
5.	Fmoc-Val
6.	Fmoc-Thr(tBu)
7.	Fmoc-Thr(tBu)
8.	Fmoc-Ala
9.	Fmoc-Arg(Pmc)
10.	Fmoc-Lys(Boc)
11.	Fmoc-Gly
12.	Fmoc-Arg(Pmc)
13.	Fmoc-Tyr(tBu)
14.	Fmoc-Gly
15.	Fmoc-Lys(Boc)
16.	Fmoc-Gly
17.	Fmoc-Asn(Trt)
18.	Fmoc-Gly
19.	Fmoc-Phe
20.	Fmoc-Met

to provide 35 mg of N-Ac-Met-Phe-Gly-Asn-Gly-Lys-Gly-Tyr-Arg-Gly-Lys-Arg-Ala-Thr-Thr-Val-Thr-Gly-Thr-Pro-NH₂ (SEQ ID NO: 42).”

Please amend page 43, line 5 – page 44, line 3 as follows:

Ac-Gln-Asp-Trp-Ala-Ala-Gln-Glu-Pro-His-Arg-His-Ser-Ile-Phe-Thr-Pro-Glu-Thr-Asn-Pro-Arg-Ala-Gly-Lcu-Glu-Lys-Asn-Tyr-NH₂ (SEQ ID NO: 43)

The title compound was prepared using the synthetic sequence described in Example 1 and using Fmoc-Tyr(^tBu) as amino acid No. 1. The following amino acids were added using the conditions indicated:

<u>No.</u>	<u>Amino Acid</u>
2.	Fmoc-Asn(Trt)
3.	Fmoc-Lys(Boc)
4.	Fmoc-Glu(γ -O ^t Bu)
5.	Fmoc-Leu
6.	Fmoc-Gly
7.	Fmoc-Ala
8.	Fmoc-Arg(Pmc)
9.	Fmoc-Pro
10.	Fmoc-Asn(Trt)
11.	Fmoc-Thr(^t Bu)
12.	Fmoc-Glu(γ -O ^t Bu)
13.	Fmoc-Pro
14.	Fmoc-Thr(^t Bu)
15.	Fmoc-Phe
16.	Fmoc-Ile
17.	Fmoc-Ser(^t Bu)
18.	Fmoc-His(Trt)
19.	Fmoc-Arg(Pmc)
20.	Fmoc-His(Trt)
21.	Fmoc-Pro
22.	Fmoc-Glu(γ -O ^t Bu)
23.	Fmoc-Gln(Trt)
24.	Fmoc-Ala
25.	Fmoc-Ala
26.	Fmoc-Trp
27.	Fmoc-Asp(β -O ^t Bu)
28.	Fmoc-Gln(Trt)

to provide 40 mg of N-Ac-Gln-Asp-Trp-Ala-Ala-Gln-Glu-Pro-His-Arg-His-Ser-Ile-Phe-Thr-Pro-Glu-Thr-Asn-Pro-Arg-Ala-Gly-Leu-Glu-Lys-Asn-Tyr-NH₂ (SEQ ID NO: 43).”

Please amend page 44, line 5 – line 9 as follows:

“N-Ac-Arg-Asn-Pro-Asp-Gly-Val-Gly-Pro-Trp-NH₂ (SEQ ID NO: 44)

The title compound was prepared using the synthetic sequence described in Example 1 and using Fmoc-Trp as amino acid No. 1. The following amino acids were added using the conditions indicated:

- | <u>No.</u> | <u>Amino Acid</u> |
|------------|--|
| 2. | Fmoc-Pro |
| 3. | Fmoc-Gly |
| 4. | Fmoc-Gly |
| 5. | Fmoc-Val |
| 6. | Fmoc-Asp(β -O ^t -Bu) |
| 7. | Fmoc-Gly |
| 8. | Fmoc-Asp(β -O ^t -Bu) |
| 9. | Fmoc-Pro |
| 10. | Fmoc-Asn(Trt) |
| 11. | Fmoc-Arg(Pmt) |

to provide 20 mg of N-Ac-Arg-Asn-Pro-Asp-Gly-Asp-Val-Gly-Gly-Pro-Trp-NH₂ (SEQ ID NO: 44).

Please amend page 44, line 12 – page 45, line 2 as follows:

“N-Ac-Tyr-Thr-Thr-Asn-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-NH₂ (SEQ ID NO: 45)

The title compound was prepared using the synthetic sequence described in example 1 and using Fmoc-Tyr(^tBu) as amino acid No. 1. The following amino acids were added using the conditions indicated:

- | <u>No.</u> | <u>Amino Acid</u> |
|------------|--|
| 2. | Fmoc-Asp(β - O ^t Bu) |
| 3. | Fmoc-Tyr(^t Bu) |
| 4. | Fmoc-Leu |
| 5. | Fmoc-Lys(Boc) |
| 6. | Fmoc-Arg(Pmc) |
| 7. | Fmoc-Pro |
| 8. | Fmoc-Asn(Trt) |
| 9. | Fmoc-Thr(^t Bu) |
| 10. | Fmoc-Thr(^t Bu) |
| 11. | Fmoc-Tyr(^t Bu) |

to provide 10 mg of N-Ac-Tyr-Thr-Thr-Asn-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-NH₂ (SEQ ID NO: 45).

Please amend page 45, line 4 – line 9 as follows:

“N-Ac-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-NH₂ (SEQ ID NO: 46)

The title compound was prepared using the synthetic sequence described in Example 1 and using Fmoc-Tyr(^tBu) as amino acid No. 1. The following amino acids were added using the conditions indicated:

- | <u>No.</u> | <u>Amino Acid</u> |
|------------|---------------------------------------|
| 2. | Fmoc-Asp(β -O ^t Bu) |
| 3. | Fmoc-Tyr(^t Bu) |
| 4. | Fmoc-Leu |
| 5. | Fmoc-Lys(Boc) |
| 6. | Fmoc-Arg(Pmc) |
| 7. | Fmoc-Pro |

to provide N-Ac-Pro-Arg-Lys-Leu-Tyr-Asp-Tyr-NH₂ (SEQ ID NO: 46) (4 mg). MS (FAB) m/z 995 (M+H)⁺.”

Please amend page 45, line 6 – line 15 as follows:

“N-Ac-Pro-Arg-Lys-Leu-Tyr-Asp-NH₂ (SEQ ID NO: 47)

The title compound was prepared using the synthetic sequence described in Example 1. The following amino acids were added using the conditions indicated:

- | <u>No.</u> | <u>Amino Acid</u> |
|------------|----------------------------|
| 2. | Fmoc-Tyr(^t Bu) |
| 3. | Fmoc-Leu |
| 4. | Fmoc-Lys(Boc) |
| 5. | Fmoc-Arg(Pmc) |
| 6. | Fmoc-Leu |

to provide N-Ac-Pro-Arg-Lys-Leu-Tyr-Asp-NH₂ (SEQ ID NO: 47) (6 mg). MS (ESI) m/z 832 (M+H)⁺.”

Please amend page 45, line 18 – page 46, line 7 as follows:

“N-Ac-Pro-Glu-Lys-Arg-Tyr-Asp-Tyr-NH₂ (SEQ ID NO: 39)

The title compound was prepared using the synthetic sequence described in Example 1 and using Fmoc-Tyr(^tBu) as amino acid No. 1. The following amino acids were added using the conditions indicated:

- | <u>No.</u> | <u>Amino Acid</u> |
|------------|---------------------------------------|
| 2. | Fmoc-Asp(β -O ^t Bu) |

3. Fmoc-Tyr(^tBu)
4. Fmoc-Arg(Pmc)
5. Fmoc-Lys(Boc)
6. Fmoc-Glu
7. Fmoc-Pro

to provide N-Ac-Pro-Glu-Lys-Arg-Tyr-Asp-Tyr-NH₂ (SEQ ID NO: 39) (6 mg). MS (FAB) m/z (1101) (M+H)⁺.”

Please amend page 46, line 10 – line 20 as follows:

“N-Ac-Arg-Lys-Leu-Tyr-Asp-Tyr-NH₂ (SEQ ID NO: 48)

The title compound was prepared using the synthetic sequence described in Example 1 and using Fmoc-Tyr(^tBu) as amino acid No. 1. The following amino acids were added using the conditions indicated:

- | <u>No.</u> | <u>Amino Acid</u> |
|------------|---------------------------------------|
| 2. | Fmoc-Asp(β -O ^t Bu) |
| 3. | Fmoc-Tyr(^t Bu) |
| 4. | Fmoc-Leu |
| 5. | Fmoc-Lys(Boc) |
| 6. | Fmoc-Arg(Pmc) |

to provide N-Ac-Arg-Lys-Leu-Tyr-Asp-Tyr-NH₂ (SEQ ID NO: 48) (8 mg). MS (ESI) m/z 898 (M+H)⁺.”

Please amend page 46, line 23 – line 35 as follows:

“N-Ac-Pro-Arg-Lys-Leu-3-I-Tyr-Asp-Tyr-NH₂ (SEQ ID NO: 4 ~~43~~)

The title compound was prepared using the synthetic sequence described in Example 1 and using Fmoc-Tyr(^tBu) as amino acid No. 1. The following amino acids were added using the conditions indicated:

- | <u>No.</u> | <u>Amino Acid</u> |
|------------|---------------------------------------|
| 2. | Fmoc-Asp(β -O ^t Bu) |
| 3. | Fmoc-3-I-Tyr(^t Bu) |
| 4. | Fmoc-Leu |
| 5. | Fmoc-Lys(Boc) |
| 6. | Fmoc-Arg(Pmc) |
| 7. | Fmoc-Pro |

to provide N-Ac-Pro-Arg-Lys-Leu-3-I-Tyr-Asp-Tyr-NH₂ (SEQ ID NO: 6) (2 mg). MS (ESI) m/z (1121) (M+H)⁺.”

Please amend page 47, line 1 – line 13 as follows:

“N-Ac-Pro-Arg-Lys-Leu-Tyr-Asp-3-I-Tyr-NH₂ (SEQ ID NO: 18 44)

The title compound was prepared using the synthetic sequence described in Example 1 and using Fmoc-3-I-Tyr(^tBu) as amino acid No. 1. The following amino acids were added using the conditions indicated:

- | <u>No.</u> | <u>Amino Acid</u> |
|------------|---------------------------------------|
| 2. | Fmoc-Asp(β -O ^t Bu) |
| 3. | Fmoc-Tyr(^t Bu) |
| 4. | Fmoc-Leu |
| 5. | Fmoc-Lys(Boc) |
| 6. | Fmoc-Arg(Pmc) |
| 7. | Fmoc-Pro |

to provide N-Ac-Pro-Arg-Lys-Leu-Tyr-Asp-3-I-Tyr-NH₂ (SEQ ID NO: 18) (2.5 mg). MS (ESI) m/z 1121 (M+H)⁺.”

Please amend page 47, line 16 – line 24 as follows:

“N-Ac-Lys-Leu-Tyr-Asp-NH₂ (SEQ ID NO: 49)

The title compound was prepared using the synthetic sequence described in Example 1 and using Fmoc-Asp(β -O^tBu) as amino acid No. 1. The following amino acids were added using the conditions indicated:

- | <u>No.</u> | <u>Amino Acid</u> |
|------------|----------------------------|
| 2. | Fmoc-Tyr(^t Bu) |
| 3. | Fmoc-Leu |
| 4. | Fmoc-Lys |

to provide 2 mg of N-Ac-Lys-Leu-Tyr-Asp-NH₂ (SEQ ID NO: 49) (2 mg).”

Please amend page 47, line 28 - page 48, line 7 as follows:

“Preparation and separation of a mixture N-Ac-Pro-Arg-Lys-Leu-Tyr-Asp-3-I¹²⁵-Tyr⁵³⁵-NH₂ (SEQ ID NO: 18) and N-Ac-Pro-Arg-Lys-Leu-3-I¹²⁵-Tyr⁵³³-Asp-Tyr-NH₂ (SEQ ID NO: 6) and (SEQ NO: ID: 18), respectively.

To a solution of 30 μ g of N-acetyl-prolyl-arginyl-lysyl-leucyl-tyrosyl-aspartyl-tyrosylamide in 80 mL of phosphate buffered saline (PBS) was added one iodobead (Pierce, Rockford, IL) and 100

μCi of NaI^{125} . After 10 minutes, the excess NaI^{125} reagent was removed by applying the reaction mixture to a Waters C18-Light SepPack column and eluting with water then 0.1% TFA in 1:1 CH_3CN /water and collecting 3 X 200 μL fractions to provide a mixture of Tyr⁵³³- and Tyr⁵³⁵- radiolabeled peptides.

The hot peptide mixture was coinjected onto a C18 HPLC column with an equimolar solution of cold carriers N-Ac-Pro-Arg-Lys-Leu-Tyr-Asp-3-I-Tyr-NH₂ (SEQ ID NO: 18) and N-Ac-Pro-Arg-Lys-Leu-3-I-Tyr-Asp-Tyr-NH₂ (SEQ ID NO: 6), the elution times of which had been predetermined as 36 and 38 minutes, respectively. Repeated elutions with the solvent system in Example 1 and lyophilization of the combined, relevant fractions provided the desired compound N-Ac-Pro-Arg-Lys-Leu-Tyr-Asp-3-I-Tyr-NH₂ (SEQ ID NO: 18) with a minimal impurity N-Ac-Pro-Arg-Lys-Leu-3-I-Tyr-Asp-Tyr-NH₂ (SEQ ID NO: 6)."

Please amend page 48, line 13 – 32 as follows:

"The kringle 5 peptide fragments were prepared from the digestion of Lys plasminogen (Lys-HPg, Abbott® Laboratories, Abbott Park, IL) with porcine elastase (SIGMA®, St. Louis, MO) by a modification of the method of Powell *et al.* (Arch Biochem. Biophys. **248**(1): 390-400 (1986), which is hereby incorporated herein by reference). 1.5 mg of porcine elastase was incubated with 200 mg of Lys-HPg in 50 mM Tris-HCl pH 8.0 and rocked overnight at room temperature. The reaction was terminated by the addition of DPF (diisopropyl fluorophosphate, SIGMA®) to a final concentration of 1 mM. The mixture was rocked for an additional 30 minutes, dialysed against 50 mM Tris pH 8.0 overnight and concentrated. The cleaved plasminogen was placed over a 2.5 cm X 15 cm lysine-Sepharose® 4B column (Brockway, W.J. and Castellino, F.J., *Arch. Biochem. Biophys.* **151**: 194-199 (1972), which is hereby incorporated by reference) and equilibrated with 50 mM Tris pH 8.0 until an absorbance of 0.05 (at 280 nm) was reached. (This step was performed to remove any fragments containing a kringle 1 region and/or a kringle 4 region (both of which bind lysine)). The non-absorbed kringle 5 peptide fragments were dialysed against 50 mM Na₂PO₄ buffer, pH 5.0 then applied to a BioRad® Mono-S column equilibrated with the same buffer. The cleaved kringle 5 portion, uncut mini-HPg and remaining protease domain fraction were eluted with a 0-20%, 20-50% and 50-70% step gradient of 20 mM Phosphate/1 M KCl pH 5.0. The kringle 5 peptide fragments eluted at the 50% step as determined by gel electrophoresis. The collected peak was dialysed overnight against 20 mM Tris pH 8.0."

Please amend page 49, line 4 – line 16 as follows:

"The *in vitro* proliferation of endothelial cells was determined as described by Lingen, *et al.* in *Laboratory Investigation*, **74**: 476-483 (1996), which is hereby incorporated herein by reference, using the Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay kit (Promega® Corporation,

Madison, WI). Bovine capillary (adrenal) endothelial cells were plated at a density of 1000 cells per well in a 96-well plate in Dulbecco's Modified Eagle Medium (DMEM) containing 10% donor calf serum and 1% BSA (bovine serum albumin, GIBCO® BRL, Gaithersburg, MD). After 8 hours, the cells were starved overnight in DMEM containing 0.1% BSA then re-fed with media containing specified concentrations of inhibitor and 5 ng/mL bFGF (basic fibroblast growth factor). The results of the assay were corrected both for unstimulated cells (i.e. no bFGF added) as the baseline and for cells stimulated with bFGF alone (i.e. no inhibitor added) as the maximal proliferation. When multiple multiple experiments were combined, the results were represented as the percent change in cell number as compared to bFGF alone."

Please amend page 49, lines 20 – 37 as follows:

"The endothelial cell migration assay was performed essentially as described by Polverini, P.J. *et al.*, *Methods Enzymol.* **198**: 440-450 (1991), which is hereby incorporated herein by reference. Briefly, bovine capillary (adrenal) endothelial cells (BCE, supplied by Judah Folkman, Harvard University Medical School) were starved overnight in DMEM containing 0.1% bovine serum albumin (BSA). Cells were then harvested with trypsin and resuspended in DMEM with 0.1% BSA at a concentration of 1.5×10^6 cells/mL. Cells were added to the bottom of a 48-well modified Boyden chamber (Nucleopore Corporation, Cabin John, MD). The chamber was assembled and inverted, and cells were allowed to attach for 2 hours at 37°C to polycarbonate chemotaxis membranes (5 μ m pore size) that had been soaked in 0.1% gelatin overnight and dried. The chamber was then reinverted and test substances were added to the wells of the upper chamber (to a total volume of 50 μ L); the apparatus was then incubated for 4 hours at 37°C. Membranes were recovered, fixed and stained (DiffQuick, Fisher Scientific®, Pittsburgh, PA) and the number of cells that had migrated to the upper chamber per 10 high power fields were counted. Background migration to DMEM + 0.1% BSA was subtracted and the data reported as the number of cells migrated per 10 high power fields (400X) or when results from multiple experiments were combined, as the percent inhibition of migration compared to a positive control. The results are shown in Table 1."

Please amend Table 1 on page 51 to read as follows:

"Table 1

Protein Fragment from SEQ ID NO: 1	Antiproliferative Activity of BCE Cells (ED ₅₀)	Migratory Inhibition of HMVEC Cells (ED ₅₀)
kringles 1-4 (angiotatin)*	135 nM	160 nM
kringle 1 (Tyr ⁸⁰ -Glu ¹⁶³)*	320 nM	-

kringle 2 (Glu ¹⁶¹ -Thr ²⁴⁵)*	no activity	-
kringle 3 (Thr ²⁵³ -Ser ³³⁵)*	460 nM	-
kringle 4 (Val ³⁵⁴ -Val ⁴⁴³)*	no activity	-
kringles 1-3 (Tyr ⁸⁰ -Pro ³⁵³)*	75 nM	60 nM
kringles 2-3 (Glu ¹⁶¹ -Ser ³³⁵)*	-	-
kringle 5 (Val ⁴⁴³ -Ala ⁵⁴³)	250 pM	200 pM
kringle 5 (Val ⁴⁴⁹ -Ala ⁵⁴³)	-	240 pM
kringle 5 (Val ⁴⁵⁴ -Ala ⁵⁴³)	-	220 pM
kringle 5 (Val ⁴⁴³ -Phe ⁵⁴⁶)	60 nM	55 nM
kringle 5 (Val ⁴⁴⁹ -Phe ⁵⁴⁶)	-	-
kringle 5 (Val ⁴⁵⁴ -Phe ⁵⁴⁶)	-	-
kringles 4-5 (Val ³⁵⁵ -Ala ⁵⁴³)	-	280 pM
kringles 4-5 (Val ³⁵⁵ -Phe ⁵⁴⁶)	-	-
N-Ac-Val ⁴⁴⁹ -Asp ⁴⁶¹ -NH ₂	-	> 1 mM
N-Ac-Met ⁴⁶³ -Pro ⁴⁸² -NH ₂	-	> 1 mM
N-Ac-Gln ⁴⁸⁴ -Tyr ⁵¹¹ -NH ₂	-	>100 μM
N-Ac-Arg ⁵¹³ -Trp ⁵²³ -NH ₂	-	500 pM
N-Ac-Tyr ⁵²⁵ -Tyr ⁵³⁵ -NH ₂	-	200 pM
N-Ac-Pro ⁵²⁹ -Tyr ⁵³⁵ -NH ₂	-	120 pM
N-Ac-Pro ⁵²⁹ -Asp ⁵³⁴ -NH ₂	-	123 pM
N-Ac-Pro ¹⁵⁰ -Tyr ¹⁵⁶ -NH ₂	-	160 nM
N-Ac-Arg ⁵³⁰ -Tyr ⁵³⁵ -NH ₂	-	80 pM
N-Ac-Pro-Arg-Lys-Leu-3-I-Tyr-Asp-Tyr-NH ₂ (SEQ ID NO: 6)	-	> 100 nM
N-Ac-Pro-Arg-Lys-Leu-Tyr-Asp-3-I-Tyr-NH ₂ (SEQ ID NO: 18)	-	400 pM
N-Ac-Lys ⁵³¹ -Asp ⁵³⁴ -NH ₂	-	-

Please amend page 52, line 5 – page 53, line 23 as follows:

“A. Production of cDNAs Encoding Kringle 5 Fragments by PCR: PCR was employed to generate cDNA fragments which encode kringle 5 peptide fragments having amino acid sequences from (1) amino acid positions 450-543 of SEQ ID NO:1 (hereinafter, K5A),

(2) amino acid positions 450-546 of SEQ ID NO:1 (hereinafter K5F), (3) amino acid positions 355-543 of SEQ ID NO:1 (hereinafter K4-5A), and (4) amino acid positions 355-546 of SEQ ID NO:1 (hereinafter K4-5F) for cloning and expression in both eukaryotic and prokaryotic hosts. DNA fragments were generated using a cDNA encoding human plasminogen (obtained from Dr. E. Reich, State University of New York, Stony Brook, NY) as template and sets of forward and reverse primers (obtained from Operon Technologies, Inc. Alameda, CA) shown below:

5'- ATTAATGGATCCTTGGACAAGAGGCTGCTTCCAGATGT AGAGACT-3'	SEQ ID NO:2
5'- ATTAATGGATCCTTGGACAAGAGGGTCCAGGACTGCTA CCATGGT-3'	SEQ ID NO:3
5'- ATTAATCTCGAGGCATGCTTAGGCCGCACACTGATGGA CA-3'	SEQ ID NO:4
5'- ATTAATCTCGAGGCATGCTTAAATGAAGGGGCCGCAC ACT-3'	SEQ ID NO:5

PCR amplifications were performed using primer sets SEQ ID NO:2 and SEQ ID NO:4 (for K5A), SEQ ID NO:2 and SEQ ID NO:5 (for K5F), SEQ ID NO:3 and SEQ ID NO:8 (for K4-5A) and SEQ ID NO:3 and SEQ ID NO:5 (for K4-5A) under standard PCR conditions, i.e. in a total reaction volume of 100 μ L containing 200 μ M of each dNTP wherein N was A, T, G and C, 0.2 μ M of each primer, approximately 10 ng of template DNA and 1 unit of Vent® DNA polymerase (New England Biolabs®). Amplifications were carried out for a total of 25 cycles (1 cycle = 94°C for one minute, 48°C for two minutes, 72°C for 1 minute) on a DNA Thermal Cycler 480 (Perkin Elmer®, Foster City, CA). After amplification, PCR products were gel purified, digested with *Bam*HI and *Xba*II (New England Biolabs®), ligated to a modified *Pichia* expression vector (pHil-D8, see below) cut with the same enzymes and transformed into HB101 cells (BioRad®) by electroporation. DNA was prepared from individual clones and subjected to restriction enzyme digestion and sequence analysis to identify clones that contained inserts with the correct sequence and in the proper orientation. Plasmids from positively identified clones were then transformed into *Pichia pastoris* strain GS115 (Invitrogen®, Carlsbad, CA) in accordance with the manufacturer's directions. To identify positive clones in *Pichia*, cells were grown in 5 mL of BMGY medium (Invitrogen®) at 29°C overnight, collected by centrifugation and resuspended in 0.5 mL

BMMY medium (Invitrogen®) for expression. After incubation at 29°C for two days, culture supernatants were collected and aliquots subjected to SDS-PAGE and western blot analysis according to known techniques. An SDS-PAGE gel is shown in FIG. 6.

B. Construction of Expression Vector pHil-D8: The *Pichia* expression vector, pHil-D8, was constructed by modification of vector pHil-D2 (Invitrogen®) to include a synthetic leader sequence for secretion of a recombinant protein (see FIG. 5). The leader sequence, 5'-

ATGTTCTCTCCAAATTTTGTCCCTTGGAATTTATTTTAGCTTTGGCTACTTTGCAATCTGT
CTTCGCTCAGCCAGTTATCTGCACTACCGTTGGTTCCGCTGCCGAGGGATCC-3'

(SEQ ID NO:9) encodes a PHO1 secretion signal (single underline) operatively linked to a pro-peptide sequence (bold highlight) for KEX2 cleavage. To construct pHil-D8, PCR was performed using pHil-S1 (Invitrogen®) as template since this vector contains the sequence encoding PHO1, a forward primer (SEQ ID NO:7) corresponding to nucleotides 509-530 of pHil-S1 and a reverse primer (SEQ ID NO:8) having a nucleotide sequence which encodes the latter portion of the PHO1 secretion signal (nucleotides 45-66 of SEQ ID NO:9) and the pro-peptide sequence (nucleotides 67-108 of SEQ ID NO:9). The primer sequences (obtained from Operon Technologies, Inc. Alameda, CA) were as follows:

5'-GAAACTTCCAAAAGTCGCCATA-3'	SEQ ID NO:7
5'- ATTAATGAATTCTCGAGCGGTCCGGGATCCCTCGGCA GCGGAACCAA CGGTAGTGCAGATAACTGGCTGAGCGAAGACAGATTGC AAAGTA-3'	SEQ ID NO:8

Amplification was performed for 25 cycles as described in Example 19. The PCR product (approximately 500 bp) was gel-purified, cut with *B/pI* and *EcoRI* and ligated to pHil-D2 cut with the same enzymes. The DNA was transformed into *E. coli* HB101 cells and positive clones identified by restriction enzyme digestion and sequence analysis. One clone having the proper sequence was designated as pHil-D8."

Please amend page 53, line 28 – page 59, line 2 as follows:

"Restriction or other modifying enzymes as well as other reagents used were obtained from commercial sources. Primers were synthesized at Abbott® Laboratories on an automatic synthesizer by standard methods known in the art.

DNAs of kringle 5 peptide fragments were also generated by PCR amplification for cloning and expression in bacterial cells (*E. coli*). The general approach taken was to generate PCR

fragments of desired coding regions, with and without termination codons, kinase the ends, and clone the fragments directly into vectors of choice. Vector constructs were then transformed into appropriate host cells and colonies screened by PCR with vector primers to confirm the presence of an insert. To determine the orientation of an insert, PCR reactions showing insert positive clones were subjected to directional PCR using 1 vector primer and 1 insert primer.

A. Preparation of blunt-end, phosphatased vectors: A description of expression vectors useful for bacterial production of kingle 5 peptide fragments is shown in Table 2.

Table 2

Vector	Source	Restriction Enzymes	Fusion
UpET	Abbott®-modified pET21d	<i>SapI</i>	None
UpET-HTh	Abbott®-modified pET21d	<i>SapI</i>	N-Terminal His6-Thrombin recognition
UpET-Ubi	Abbott®-modified pET21d	<i>SapI</i>	N-Terminal His6-Ubiquitin-Enterokinase recognition
pET32a	Novagen®	<i>NcoI</i> + <i>XbaI</i>	Thioredoxin, Enterokinase recognition
pGEX-4T-2	Pharmacia®	<i>EcoRI</i> + <i>NcoI</i>	GST
pCYB3	New England Biolabs®	<i>NcoI</i> + <i>SapI</i>	C-terminal intein

All vectors were first isolated and purified using Qiagen® columns in accordance with the manufacturer's instructions (QIAGEN®, Inc., Santa Clarita, CA). Vector DNA (1 µg) was digested with appropriate restriction enzymes (see Table 2) in 20 µL of NEB4 buffer (New England Biolabs®) containing 100 µg/mL bovine serum albumin (BSA). The reaction was centrifuged briefly, 20 µL of deionized H₂O, 0.4 µL of dNTP mix (Pharmacia®; 20 mM each dNTP) and 0.25 µL of cloned *pfu* DNA polymerase (Stratagene®; 2.5 units/µL) was added and the reaction mixture incubated at 65°C for 20 minutes to fill in the vector ends. The reaction mixture was again centrifuged briefly and 4 µL of diluted calf intestinal phosphatase (GIBCO® BRL, Gaithersburg, MD; 5 units total) was added. The mixture was then incubated at 50°C for one hour. Five 5 µL of 10%SDS, 2 µL of 5 M NaCl, 2 µL of 0.5 M EDTA and 45 µL of H₂O were added, the reaction was centrifuged briefly and then incubated at 65°C for 20 minutes. The reaction was then extracted

three times with buffer-saturated phenol-chloroform (GIBCO® BRL) and once with chloroform. The aqueous phase was purified through a CHROMA SPIN™ 1000 TE column (CLONTECH®, Palo Alto, CA).

B. Generation of DNA Fragments by PCR: PCR primers were designed and ordered based upon the published sequence for human plasminogen (see SEQ ID NO:12) and are shown below:

5'-GTCCAGGACTGCTACCAT-3'	SEQ ID NO:10
5'-CTGCTTCCAGATGTAGAGA-3'	SEQ ID NO:11
5'-TTATTAGCCGCACACTGAGGGA-3'	SEQ ID NO:13

Unless otherwise noted, all PCRs were performed with *pfu* DNA polymerase and buffer (Stratagene®), using 200 µM each dNTP and 1 µM each primer. Primer sets used were SEQ ID NO:11 and SEQ ID NO:13 (for K5A), and SEQ ID NO:10 and SEQ ID NO:13 (for K4-5A). Vector pHis-D8 containing the K4-K5A (described in Example 19) was used as template. Prior to use as a template, this DNA was digested with *DraI* (which makes multiple cuts outside of the kringle regions) in order to eliminate background due to the pHis-D8 vector in transformations. Approximately 10 ng of template was used per 50 µL PCR reaction. PCR reactions were run at 94°C for 2 mins.; then for 15 cycles of 94°C, 30 sec.; 49°C, 1 min.; 72°C, 4 mins; and 72°C, 7 mins. After the PCR reaction, 0.5 µL of 100 mM ATP and 5 units of T4 Kinase was added and the reaction incubated at 37°C for 20 mins. to kinase the ends. The reaction was then heated at 68°C for 15 mins. and purified over an S400-HR spin column (Pharmacia®) for use in ligations.

C. Ligation of PCR Fragments into Expression Vectors: Six recombinant constructions (specifically, (i) K5A in UpET-PS3, (ii) K5A in pET32a, (iii) K4-5A in UpET-PS3, (iv) K4-5A in UpET-Ubi, (v) K4-5A in pET32a and (vi) K4-5A in pGEX-4T-2) were made as follows: blunt-end, phosphatased vector (1 µL from step A above) and PCR fragment (1 µL from step B above) were ligated in a total volume of 5.5 µL using a Rapid Ligation Kit as per the manufacturers instructions (BOEHRINGER-MANNHEIM® Corp., Indianapolis, IN). Ligation mixture (1 µL) was then transformed into 20 µL of competent cells (XL1-Blue Supercompetent cells or XL2-Blue Ultracompetent cells (Stratagene®)) as per the manufacturer's instructions. Recombinant cells were selected on LB-Amp agar plates (MicroDiagnostics, Lombard, IL).

D. Expression Studies: pGEX vectors were expressed in *E. coli* XL1-Blue or XL2-Blue. All other vectors were isolated and retransformed into *E. coli* BL21(DE3) (Novagen®) as per the manufacturer's instructions. Individual colonies were inoculated into 2.5 mL of LB/Amp and shaken at 225 rpm, 37°C, overnight. Overnight culture (0.5 mL) was then inoculated into 50 mL of LB/Amp in a 250 mL flask and shaken at 225 rpm, 37°C to an OD600 of 0.5 - 0.6. Isopropyl-1-

thio- β -D-galactopyranoside (IPTG, 100 mM) was then added to a final concentration of 1 mM. The culture was shaken at 225 rpm, 30°C for 3 hours before being spun down. Samples were prepared for SDS-PAGE in accordance with known techniques. Preliminary experiments showed that cells having K5A/pET32a, K4-5A/pET32a and K4-5A/pGEX produced the most recombinant protein. Cultures of these clones was then analyzed for soluble vs. insoluble expression by SDS-PAGE. As FIG. 7 shows, K5A/pET32a produced recombinant protein that is almost completely soluble (compare lanes S and P of Trx-K5A), whereas K4-5A/pET32a and K4-5A/pGEX produced about 75% soluble protein.

E. Construction of Abbott®-modified vectors

i. VB1, VB2, VB3 and VB4 Cassette Preparation: VB1, VB2, VB3, and VB4 were made as synthetic DNAs using techniques well known to those of ordinary skill in the art. The sequences of synVB1, synVB2, synVB3, and synVB4 are shown below:

synVB1	5'- AGCGTCTCATGAAGAGCTGGCTCACCTTCGGGTGGGC CTTTCTGCGCCTTGGCGCGCCAACCTTAATTAACCGGG AGCCCGCCTAATGAGCGGGCTTTTTTTTGCTCTTCATA GTGACTGAGACGTCG-3'	SEQ ID NO:14
synVB2	5'- AGCGTCTCAGGTGGTGGTCATCACCATCACCATCACGG TGGTGGTCTGGTGGCGCGCGGCAAGCTGAAGAGCTGGC TCACCTTCGGGTGGGCCTTTCTGCGCCTTGGCGCGCCA ACCTTAATTAACCGGGAGCCCGCCTAATGAGCGGGCTT TTTTTTGCTCTTCACGAGACGTCG-3'	SEQ ID NO:15
synVB3	5'- AGCGTCTCAGGTGGTGGTCATCACCATCACCATCACGG TGGTGGTTGAAGAGCTGGCTCACCTTCGGGTGGGCCT TTCTGCGCCTTGGCGCGCCAACCTTAATTAACCGGGAG CCCGCCTAATGAGCGGGCTTTTTTTTGC TCTTCACGAGACGTC-3'	SEQ ID NO:16
synVB4	5'- AGCGTCTCAGGTGGTGGTCATCACCATCACCATCACGG TGGTGGTGATGACGATGACAAGTGAAGAGCTGGCTCA CCTTCGGGTGGGCCTTTCTGCGCCTTGGCGCGCCAACC TTAATTAACCGGGAGCCCGCCTAATGAGCGGGCTTTTT TTTGCTCTTCACGAGACGTCG-3'	SEQ ID NO:17

Each synthetic sequence was made double stranded and cloned into pCR-Script Cam™ (Stratagene®) as per the manufacturer's instructions; clones with the correct sequence were then isolated by standard procedures. Five µg of purified DNA was digested with 8 units of *BsmBI* at 55°C in 20 µL reactions in 1X NEB4 Buffer containing 100 µg/mL BSA. The reaction was centrifuged briefly, 20 µL of deionized H₂O, 0.4 µL of dNTP mix (Pharmacia®; 20 mM each dNTP) and 0.25 µL cloned *pfu* DNA polymerase (Stratagene®; 2.5 units per µL) were added and the reaction was incubated at 65°C for 20 minutes to fill in the ends. The DNA was then run on a 3% MetaPhor™ Agarose gels (FMC, Rockland, Maine) in 0.5X Tris-Acetate-EDTA buffer (TAE). The cassette band was cut out and the DNA was eluted by freezing the gel and centrifuging the buffer through an Ultrafree™ Probind cartridge (MILLIPORE® Corp., Bedford, MA), followed by isopropanol precipitation using Pellet Paint™ (Novagen®) as a carrier. The DNA (cfVB1, cfVB2, cfVB3, and cfVB4) was rinsed with 70% ethanol, dried briefly and resuspended in 25µL of Tris-EDTA (TE) buffer.

ii. Construction of UpET: Vector pET21d (Novagen®) was digested with *SapI*, treated first with T4 DNA Polymerase + dGTP, then Mung Bean Nuclease, then DNA polymerase I Klenow fragment and religated. Individual colonies were screened to select a plasmid in which the existing *SapI* site had been eliminated. This DNA was then digested with *NotI* + *BamHI* and ligated to 5'-CATGTGAAGAGC-3' (SEQ ID NO:19) + 5'-GATCGCTCTTCA-3' (SEQ ID NO:20) to introduce a single *SapI* site. Purified, verified cloned DNA was cut with *SapI* + *HindIII*, blunted and phosphatased as described above, ligated with the cfVB1 cassette, transformed into *E. coli* and plated on LB-Amp plates. Colonies were picked with sterile pipette tips onto LB-Amp agar plates and into 20 µL of AmpliTaq® PCR mix (Perkin Elmer®) in Costar Thermowell plates containing 1 µM of each vector primers 5'-AGATCTCGATCCCGCGAA-3' (forward primer, SEQ ID NO:21) and 5'-ATCCGGATATAGTTCCTC-3' (SEQ ID NO:22). Reactions were heated to 94° for 5', then cycled using a GeneAmp 9600 thermal cycler for 30 cycles of 94°, 30 seconds; 40°, 1 minute; 72°, 2 minutes. 10 µL of each reaction was run on agarose gels. To determine the orientation of the cassette, 0.25 µL of a PCR screen with the correct size was added to a fresh reaction containing the reverse vector primer and a cassette primer 5'-CGGGCTTTTTTTTGCCTCTCA-3' (SEQ ID NO:23). Reactions were cycled as above for an additional 10 cycles. Final vectors were sequenced using standard procedure and one clone designated as UpET.

iii. Construction of UpET-HTh: UpET was digested with *SapI* and prepared for blunt, phosphatased cloning. It was ligated to the cfVB2 cassette, transformed, colonies screened and sequenced as for the cfVB1 ligation above.

iv. Construction of UpET-H: UpET was digested with *SapI* and prepared for blunt, phosphatased cloning. It was ligated to the cVB3 cassette, transformed, colonies screened and sequenced as for the cVB1 ligation above.

v. Construction of UpET-Ubi: A PCR fragment for *S. cerevisiae* ubiquitin was generated using Ulta DNA polymerase and buffer (Perkin Elmer®), 40 µM each dNTP, 1 µM each of the primers 5'-CAGATTTTCGTCAAGACTT-3' (Ubi-5p, SEQ ID NO:24) and 5'-ACCACCTCTTAGCCTTAG-3' (Ubi-3p, SEQ ID NO:25) and 1.75 µg of yeast DNA at 94°C, 2 mins. then 25 cycles of 94°C, 1 min.; 40°C, 1 min.; 72°C, 2 mins.; then 72°C for 7 mins. A PCR fragment was generated from 20 ng of pET15b (Novagen®) using the primers 5'-CATGGTATATCTCCTTCTT-3' (pET3p-ATG, SEQ ID NO:26) and 5'-TGAGCAATAACTAGCATAAC-3' (T7RevTerm, SEQ ID NO:27) at 94°C, 2 mins. then 10 cycles of 94°C, 45 sec.; 42°C, 1 min.; 72°C, 15 mins.; then 72°C for 7 mins. The Ubiquitin and pET15b-derived PCR fragments were gel-purified and ligated together using BRL T4 ligase and ligase buffer. A T7 promoter-ubiquitin (T7-ubiquitin) PCR fragment was then generated using the ligation as template and Ulta DNA polymerase and the primers 5'-AGATCTCGATCCCGCGAA-3' (pET5p, SEQ ID NO:28) and SEQ ID NO:25 at 94°C, 2 mins. then 25 cycles of 94°C, 30 sec.; 42°C, 1 min.; 72°C, 3 mins.; then 72°C, 7 mins. The T7-ubiquitin PCR fragment was gel purified.

A PCR fragment for mature human Stromelysin was generated using Ulta DNA polymerase (as above) with the primer 5'-TTAGGTCTCAGGGGAGT-3' (Strom-3p, SEQ ID NO:29) and kinased primer 5'-TTCAGAACCTTTCCTGGCA-3' (Strom-5p, SEQ ID NO:30) and approximately 20 ng of template (i.e. stromelysin cloned into pET3b (Novagen®)) at 94°C, 2' then 15 cycles of 94°C, 1 min.; 44°C, 1 min.; 72°C, 2 mins., then 72°C for 7 mins.. The stromelysin PCR reaction (10µL) was ligated with 100 pMol of annealed oligos 5'-AGCGGCGACGACGACGACAAG-3' (Ek-Cut-5p, SEQ ID NO:31) and 5'-CTTGTCGTCGTCGTCGCGCT-3' (Ek-Cut-3p, SEQ ID NO:32 coding for an Enterokinase cleavage site) in 40 µL of BRL ligase and ligase buffer. An enterokinase site - mature stromelysin (Ek-Stromelysin) PCR fragment was generated using 1 µL of this ligation as a template, primers SEQ ID NO:29 and kinased SEQ ID NO:31, Ulta DNA polymerase and buffer at 94°C, 2 mins.; then 10 cycles of 94°C, 1 min.; 44°C, 1 min.; 72°C, 2 mins., then 72° for 7 mins. The Ek-Stromelysin PCR fragment was gel purified.

The T7-ubiquitin and Ek-stromelysin PCR fragments were ligated together in BRL ligase and ligase buffer. A T7-ubiquitin-Ek-stromelysin PCR fragment was then generated using the ligation as template and Ulta DNA polymerase and the primers SEQ ID NO:28 and SEQ ID NO:29 at 94°C, 2' then 25 cycles of 94°C, 30 sec.; 42°C, 1 min.; 72°C, 6 mins., then 72°C for 7 mins.

A PCR fragment was generated using the stromelysin-pET3b plasmid template with the primers SEQ ID NO:26 and SEQ ID NO:30 with KlenTaq (AB Peptides, St. Louis, MO) and *pfu*

DNA polymerases at 94°C, 2' then 15 cycles of 94°C, 30 sec.; 42°C, 2 mins.; 68°C, 20 mins.. This PCR fragment was mixed with the T7-Ubiquitin-Ek-Stromelysin PCR fragment and transformed into BRL DH5 α maximum efficiency competent cells. Correct clones were identified by isolation of plasmid DNA, transfection into BL21(DE3), and expression studied as described above.

A PCR fragment for Ubiquitin-Ek was generated from a correct T7-Ubiquitin-Ek-Stromelysin expression plasmid with the primers SEQ ID NO:24 and SEQ ID NO:32 and *pfu* DNA polymerase at 94°C, 2' then 20 cycles of 94°C, 30 sec.; 40°C, 1 min.; 72°C, 3 mins., 72°C, 7 mins. The fragment was purified over a Pharmacia® S-400 HR Spin column and ligated to the VBC1 cassette using the Rapid DNA Ligation kit. A PCR fragment was generated using the ligation as template and the primers SEQ ID NO:24 and 5'-TGAAGAGCAAAAAAAGCCCG-3' (SEQ ID NO:33) and *pfu* DNA polymerase at 94°C, 2 mins. then 20 cycles of 94°C, 30 sec.; 40°C, 1 min.; 72°C, 2 mins., 72°C, 7 mins. The PCR fragment was kinased and ligated to Upet-H prepared for blunt, phosphatased cloning. The ligation was transformed into competent cells and colonies were screened by PCR as above. Plasmid DNA was sequenced to identify correct clones of UpEt-Ubi."

Please amend page 59, line 8 – page 60, page 2 as follows:

"A. Method 1: Kringle fragment 1-5 (K1-5) was prepared from the limited digestion of Lys-HPg (Abbott® Labs) with porcine elastase (Sigma® Chemical Co., St. Louis, MO) by the method of Powell and Castellino, 1986, *supra*. Briefly, 1.5 mg of elastase was incubated with 200 mg of Lys-HPg in 50 mM Tris-HCl pH 8.0 and rocked for four hours at room temperature. The reaction was terminated by the addition of diisopropyl fluorophosphate (DFP, Sigma® Chemical Co., St. Louis, MO) to a final concentration of 1 mM. The mixture was rocked for an additional 30 minutes, dialyzed against 50 mM Tris pH 8.0 overnight and concentrated. The cleaved HPg was placed over a lysine-Sepharose 4B column (2.5 cm X 15 cm) (Brockway and Castellino, 1972) equilibrated with 50 mM Tris pH 8.0 until an absorbance at 280 nm of 0.05 was reached. The absorbed kringle fragments were then bulk eluted with 100% buffer of 50 mM Tris-HCl/300 mM ϵ -amino caproic acid, pH 8.0. After dialysis of the kringle fractions against 20 mM Tris-HCl, pH 5.0, the material was applied to a BioRad® Mono-S column equilibrated with the same buffer. The kringle portions, K4, K5, K1-3, K1-4 and K1-5 were eluted with a 0-20%, 20-50%, 50-70% and 70-100% step gradient of 20 mM phosphate to 20 mM phosphate /1 M KCl pH 5.0. K1-5 eluted at the last step gradient of the 70-100% step as determined by gel electrophoresis. The molecular weights were determined by gel electrophoresis and Western Blot analysis with monoclonal antibodies specific for kringle 4, kringle 5, kringle 1-3 fragments and the protease domain.

B. Method 2: Kringle fragment 1-5 (K1-5), was prepared from the limited digestion of

Lys-HPg (Abbott® Labs) with porcine pepsin (Sigma® Chemical Co., St. Louis, MO). The Lys-HPg was added to a slurry of insoluble pepsin-agarose containing 100 mM ε-amino caproic acid, pH 8.0 and stirred for 2 hours. The mixture was centrifuged at 3000 rpm to remove the pepsin agarose. The supernatant was dialysed against 50 mM PBS, pH 7.4 overnight. The sample was then placed over a lysine-Sepharose® 4B column (Sigma®)(2.5 cm X 15 cm) (Brockway and Castellino, 1972) equilibrated with 50 mM Tris pH 8.0 until an absorbance at 280 nm of 0.05 was reached. Kringle fragment K1-5 was eluted with 100% buffer of 50 mM Tris/300 mM ε-amino caproic acid, pH 8.0. The sample was determined by gel electrophoresis, and Western Blot analysis with monoclonal antibodies specific for kringle 4, kringle 5, kringle 1-3 fragments and the protease domain.”